

*Original Article***Combination treatment with an ET_A-receptor blocker and an ACE inhibitor is not superior to the respective monotherapies in attenuating chronic transplant vasculopathy in different aorta allotransplantation rat models**

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Abstract

Background. The effect of the specific endothelin_A (ET_A)-receptor antagonist LU 302146 (LU) was assessed in a normotensive model of chronic transplant vasculopathy, i.e. orthotopic allotransplantation of the infrarenal abdominal aorta from spontaneously hypertensive-to-Wistar-Kyoto (SHR-to-WKY) rats. A second experimental setting was used to confirm the results in a different model, which to some extent may also address the issue of blood pressure (BP) in transplant vasculopathy, i.e. orthotopic allotransplantation of infrarenal abdominal aorta from WKY-to-SHR rats. Untreated sham-operated and isografted WKY and SHR served as controls. Allografted animals treated with the angiotensin-converting enzyme (ACE) inhibitor trandolapril served as positive treatment controls.

Methods. Rats were randomized to receive standard diet or a diet designed to deliver either 30 mg LU/kg bw/day, 0.3 mg/kg bw/day trandolapril or a combination of both. The duration of either experiment was 8 weeks. BP was measured by tail plethysmography.

Results. Treatment with LU did not affect systolic BP in either experimental setting. In contrast, trandolapril and combination treatment significantly reduced systolic BP in SHRs. The increase in aortic wall thickness (given in mm) was abrogated to a similar extent in the three treatment groups as compared with untreated allotransplanted animals in either experimental setting (e.g. WKY sham-operated 0.084 ± 0.013 , $P < 0.05$ vs treatment groups; WKY isografted 0.100 ± 0.010 , $P < 0.05$ vs treatment

groups; WKY allotransplanted 0.289 ± 0.077 , $P < 0.05$ vs all groups; WKY allotransplanted + trandolapril 0.185 ± 0.025 ; WKY allotransplanted + LU 302146 0.192 ± 0.049 ; WKY allotransplanted + LU 302146 + trandolapril 0.190 ± 0.041). This was due to an attenuation of the increase of intima and media thickness. Treatment with LU and trandolapril were similarly effective in attenuating the increase of the number of proliferating cell nuclear antigen (PCNA)-positive cells in the intima. Again, combination treatment did not confer additional benefit. In contrast, trandolapril was more effective than LU in attenuating the increase in the number of PCNA-positive cells in the media. Trandolapril or combination treatment, but not LU, attenuated transforming growth factor- β expression in aortic allografts.

Conclusions. The ET_A-receptor blockade abrogates allograft vasculopathy in two different aorta allotransplantation models to a similar extent as ACE inhibition even in the absence of concomitant immunosuppression. At least in SHRs the effect of ET_A-receptor blockade is independent of BP. This finding is consistent with the notion that ET_A-receptor mediated events play a partly BP-independent role in the genesis of chronic transplant vasculopathy.

Keywords: ACE inhibitor; chronic allograft vasculopathy; endothelin; endothelin antagonist; endothelin receptor

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Introduction

So-called ‘chronic rejection’ is the major cause of renal and cardiac allograft loss, and no specific treatment of

this entity is available. Although chronic rejection is triggered by immune mechanisms, damage is perpetuated and amplified by non-immune mechanisms, potentially including the endothelin (ET) and renin-angiotensin systems. Recent experimental studies have shown that ET-1 gene expression and/or increased synthesis of ET-1 are found during chronic rejection of renal and cardiac allografts. Chronic rejection of rat cardiac allografts is associated with increased ET-1 expression in ventricular tissue, which is particularly pronounced in areas of intimal proliferation [1]. Expression of ET-1 is also seen in cardiac myofibers that are situated in close proximity to areas of interstitial inflammation or fibrosis [2]. The potential clinical relevance of these findings is supported by an increased expression of ET-1 in biopsy specimens of humans with cardiac or renal allografts undergoing chronic rejection [3,4].

We have recently reported that ET_A-receptor blockade and angiotensin-converting enzyme (ACE) inhibition are equally effective in attenuating the development of chronic transplant nephropathy in a 'Fisher-to-Lewis' rat model [5]. Combination treatment of both substances had no additive nephroprotective effect. In contrast to ACE inhibition, the effect of ET_A-receptor blockade was independent of blood pressure (BP). This finding is noteworthy, because BP is apparently an important non-immune mechanism promoting chronic rejection, at least of renal allografts [6]. Thus, ET_A-receptor-mediated events seem to be of pivotal, BP-independent importance in the genesis of chronic allograft rejection. BP may, however, modulate the beneficial effect of ET_A-receptor blockade.

It is therefore crucial to investigate whether our findings in a 'Fisher-to-Lewis' rat model [5] are specific for kidney transplantation or can be generalized and demonstrated for other organ transplants as well. Thus, the effect of specific ET_A-receptor blockade with LU 302146 (LU) on the development of chronic allograft vasculopathy was investigated in an orthotopic aorta allotransplantation model, i.e. transplantation of infrarenal aorta from spontaneously hypertensive-to-Wistar-Kyoto (SHR-to-WKY) rats. As a control, ACE inhibition was assessed in parallel. A parallel experiment was performed to confirm the results in another chronic transplant vasculopathy model that probably also addresses the issue of BP in the genesis of transplant vasculopathy, i.e. a hypertensive model of orthotopic allotransplantation of infrarenal abdominal aorta from WKY-to-SHR rats.

Subjects and methods

Animals

Eight-week-old male SHR ($n=72$) and WKY ($n=71$) rats weighing 180–200 g were purchased from M&B A/S (Ry, Denmark) and Iffa-Credo (Lyon, France), respectively. The rats were fed standard rat chow (0.25% NaCl and 19% protein; ssnf GmbH, Soest, Germany).

Aorta transplantation and experimental design

Aortas were transplanted by microsurgical techniques under anaesthesia with xylazine (5 mg/kg body weight) and ketamine (100 mg/kg body weight). Using the operation microscope, the infrarenal aorta was exposed from the left renal vein to the aortic bifurcation via midline laparotomy. Any aortic branches in this segment were ligated.

Two microclips were placed, one below the renal arteries and the second above the aortic bifurcation. A 1 cm segment of the aorta was removed from the donor animal and washed with saline solution. A similar resection of the infrarenal aorta was performed in the recipient rat. The graft was inserted into the aorta of the recipient by end-to-end interrupted anastomoses, using 10-0 prolene sutures. The aorta was clamped for 30 min; thereafter patency of each graft was evaluated. No acute secondary graft thrombosis was observed. Twelve SHRs and 11 WKY rats were sham-operated, including ligation of collateral vessels. Furthermore, 12 animals of either strain were isografted as additional controls. The advantage of both models investigated is that no immunosuppressive treatment is necessary.

The WKY-to-SHR allografts and SHR-to-WKY allografts with no treatment were compared with WKY-to-SHR and SHR-to-WKY allografts treated with LU, trandolapril and a combination of both (each group $n=12$). LU 302146 was added to the food calculated to deliver 30 mg/kg bw/day and trandolapril to deliver 0.3 mg/kg bw/day. This dose of the ET_A-receptor blocker was chosen because it is known to completely block ET_A-receptors without affecting ET_B-receptors [7]. LU 302146 is the follow-up molecule of LU 135252 with similar pharmacological properties (M. Raschack, personal communication). To allow comparability, the same dose of trandolapril was chosen as in our experiment in the 'Fisher-to-Lewis' chronic transplant nephropathy model [5]. Treatment was started on the first day post-operation. A pair-feeding protocol was used throughout the experiment. BP of awake rats was measured weekly by tail plethysmography. Rats were sacrificed after 8 weeks and the infrarenal aortic graft (or native aorta in sham-operated animals) was excised. The grafts were fixed in formalin for morphometric analysis and immunohistochemical studies. A few animals died shortly after operation. The final numbers of animals investigated in each group were: nine in the WKY sham-operated group, 12 in the WKY isograft group, 10 in the allotransplanted WKY group, and 11, 9 and 10 in the allotransplanted WKY animals treated with trandolapril, LU 302146, or a combination of both, respectively. There were 12 in all SHR groups, except 10 in the allotransplanted SHR group and 10 in the allotransplanted SHR treated with trandolapril groups.

Morphometric investigations

Cross sections of the aortic graft were prepared, semithin (1 μ m) sections were cut, stained with methylene blue and basic fuchsin, and studied planimetrically as described below at a magnification of $\times 80$. Using a semiautomatic image analysing system (Optimas 5.2, Bioscan, Stemmer Co., Munich, Germany), the external and internal contours of the media and intima were outlined, and the cross-sectional area, wall thickness, and minimal and maximal lumen

diameters calculated. The wall:lumen ratio was calculated by dividing the mean wall diameter by the minimal lumen diameter.

Connective tissue staining and immunohistochemistry

Staining for connective tissue was performed in formalin-fixed, paraffin-embedded sections, which were stained with Sirius. Immunohistochemistry was performed essentially as described previously [8]. Paraffin-embedded sections were deparaffinized with xylene and graded ethanol before being treated with Power Block (Biogenex, Ramon, CA, USA) for 20 min to inhibit the non-specific staining. The sections were incubated either with anti-proliferating cell nuclear antigen (anti-PCNA) antibody (1:150 dilution; Immunotech, Marseille, France) or anti-transforming growth factor- β (anti-TGF- β) antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight and were subsequently processed using a biotin-streptavidin

detection system (biotin-streptavidin super sensitive; Biogenex) with fast red substrate system (DAKO Diagnostika GmbH, Hamburg, Germany) as the chromogen. The sections were finally counterstained with haematoxylin. Examination was performed using light microscopy at a magnification of $\times 100$.

Quantitative evaluation of connective tissue staining

Connective tissue was quantified in five randomly chosen animals per group using a score for the amount of Sirius staining in the intima, media and adventitia of the aortic wall. The grading was as follows: 0, no staining; 1, little staining; 2, moderate staining; 3, widespread staining.

Statistics

Data are given as means \pm SD. Statistical analysis was performed using one-way ANOVA followed by Bonferroni-Dunn multiple range test.

Table 1. Systolic BP (mmHg, measured by tail plethysmography) in the last week of the experiments (week 8)

Groups	WKY sham	WKY iso	WKY allo	WKY allo + ACEi	WKY allo + ET _A RB	WKY allo + comb	SHR sham	SHR iso	SHR allo	SHR allo + ACEi	SHR allo + ET _A RB	SHR allo + comb
Systolic BP	120 \pm 20	100 \pm 13	111 \pm 17	103 \pm 19	118 \pm 17	108 \pm 9	166 \pm 31*	144 \pm 20*	159 \pm 24*	113 \pm 21 [†]	149 \pm 15 [‡]	128 \pm 14 [†]

WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; sham, sham-operation; iso, isortransplantation; allo, allotransplantation; ACEi, ACE inhibitor; ET_ARB, ET_A-receptor blocker; comb, combination therapy (ACEi + ET_ARB). * $P < 0.001$ vs WKY sham, iso and all allo groups; [†] $P < 0.01$ vs SHR allo; [‡] $P < 0.001$ vs SHR allo treated with ACEi monotherapy.

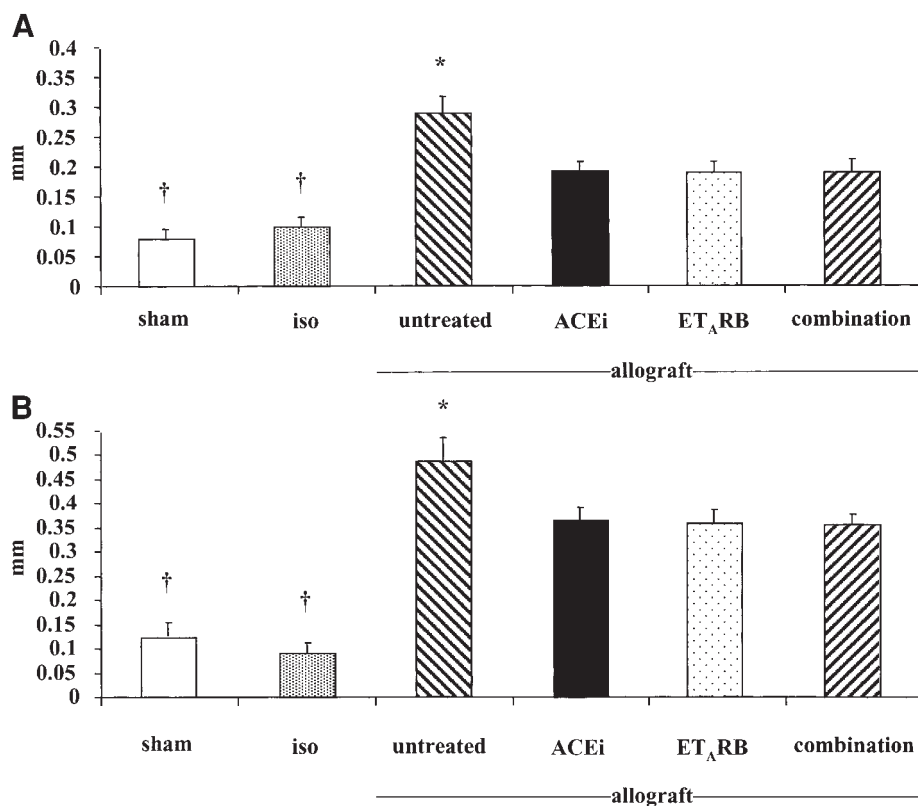


Fig. 1. Aortic wall thickness (mm) in WKY (A) and SHR rats (B). Sham, sham-operation; iso, isortransplantation; ACEi, ACE inhibitor; ET_ARB, ET_A-receptor blocker; comb, combination therapy (ACEi + ETRB). * $P < 0.01$ vs treatment groups; [†] $P < 0.01$ vs allograft groups.

Results

Animal data

Body weight and blood parameters. Body weight, haemoglobin, haematocrit, serum creatinine, serum urea, electrolytes, serum cholesterol and triglycerides did not differ between the groups in both experimental settings (data not shown).

Blood pressure. Sham-operated, iso- and allotransplanted SHR rats had higher systolic BP than the respective WKY rats ($P < 0.001$). Systolic BP was not altered in either strain after iso- and allotransplantation had been performed. Trandolapril, but not LU, significantly reduced systolic BP in allotransplanted SHR rats. Combination treatment with trandolapril plus

LU did not further decrease systolic BP in SHR rats as compared with trandolapril alone. In normotensive WKY neither of the treatments influenced systolic BP (Table 1).

Morphological studies

Morphology in isografts was virtually normal and was comparable with that of sham-operated rats in either experimental setting, i.e. SHR-to-WKY and WKY-to-SHR grafts. In sham-operated animals, aortic wall thickness was not different between SHR and WKY (Figure 1). Aortic wall thickness was significantly greater, however, in SHR recipients of WKY allografts as compared with WKY recipients of SHR allografts (Figures 1 and 3). LU attenuated the increase in wall thickness in both rat strains (Figures 1 and 2).

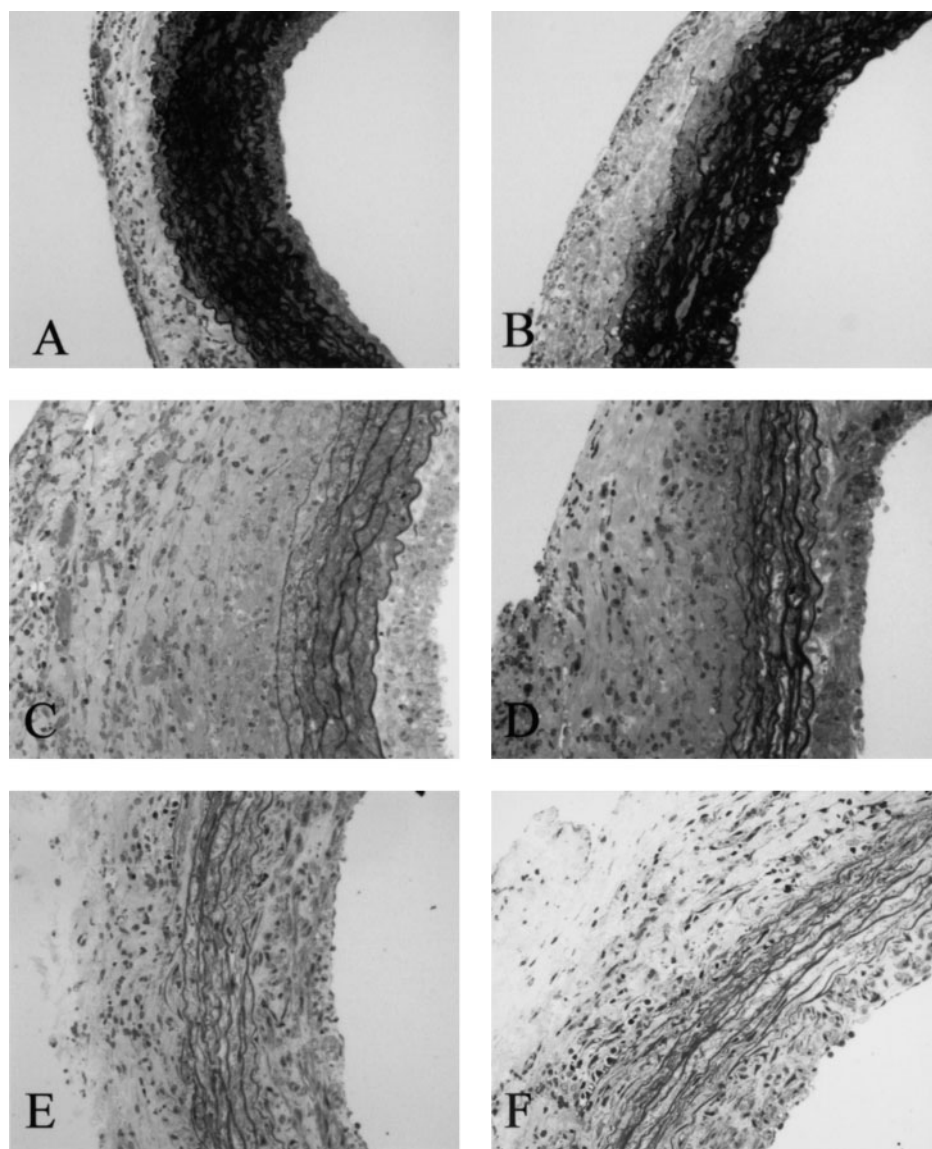


Fig. 2. Aortic wall thickness in sham-operated (A) and iso-transplanted (B) WKY animals as negative controls compared with untreated allotransplanted WKY (C), allotransplanted WKY treated with the ACE inhibitor trandolapril (D), the ET_A-receptor blocker LU 302146 (E), and a combination of ACE inhibitor and ET_A-receptor blocker (F), respectively. Semithin sections, magnification $\times 100$.

This effect was due to attenuation of the increase in media and intima thickening. The magnitude of the effect of ET_A-receptor blockade was comparable with that of ACE inhibition. There was no statistically significant difference between combination therapy and the respective monotherapies (Figures 1 and 2). These data were confirmed by measurement of the cross-sectional aortic areas and the wall:lumen ratio (data not shown).

Connective-tissue staining

Connective-tissue staining in the aortic wall was more pronounced in untreated allotransplanted animals when compared with sham-operated or isografted animals of either strain. This increase in the amount of connective tissue was similarly reduced by ACE inhibition, ET_A-receptor blockade and combination therapy (Table 2).

Immunohistochemistry

Proliferating cell nuclear antigen. The number of PCNA-positive cells per intima and media cross section was significantly higher in untreated allotransplanted animals compared with sham-operated or isografted animals in both strains. The PCNA staining was comparable in sham-operated and isografted rats. Treatment with LU and trandolapril was similarly effective in attenuating the increase in the number of PCNA-positive cells in the intima. In the media, the effect of trandolapril was

significantly more pronounced than that of LU. Combination treatment did not confer additional benefit. The results of PCNA immunohistochemistry are shown in Figure 4.

Transforming growth factor- β . Immunostaining for TGF- β was virtually negative in sham-operated and isografted animals of both strains. In contrast, TGF- β staining was strongly detectable in allotransplanted WKY and SHR animals. This increase in TGF- β staining was markedly abrogated by treatment with trandolapril, but not by treatment with LU. Combination therapy did not confer an additional benefit compared with ACE inhibition alone (Figure 5).

Discussion

The present study documents that development of chronic transplant vasculopathy in two different aorta allotransplantation models is abrogated to a similar extent by treatment with the ET_A-receptor blocker LU and the ACE inhibitor trandolapril. This result confirms that the beneficial effect of ET_A-receptor blockade is not unique to the kidney, i.e. the 'Fisher-to-Lewis' chronic transplant nephropathy model [5], and points to a more general role of the ET system in chronic allograft rejection.

The normotensive (aortic allotransplantation from SHR-to-WKY) and hypertensive (aortic allotransplantation from WKY-to-SHR) models used in our study

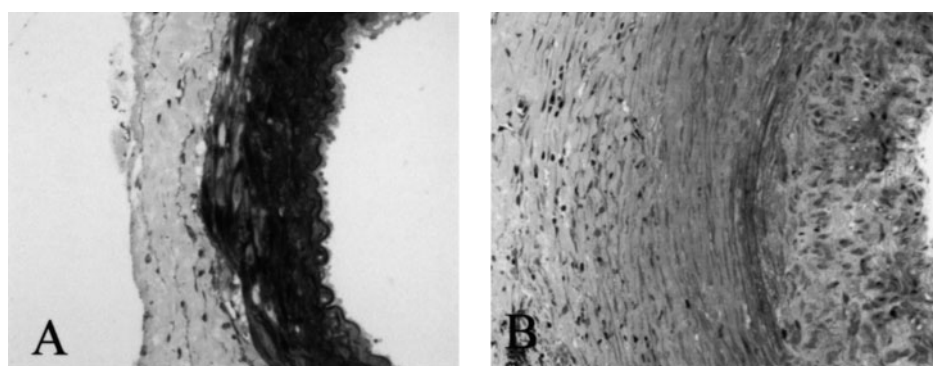


Fig. 3. Iso-transplanted aortic graft thickness in SHR (A) compared with marked thickening of aortic wall in untreated allotransplanted SHR (B). Semithin sections, magnification $\times 140$.

Table 2. Results of the quantitative evaluation of Sirius-staining for connective tissue in the intima, media and adventitia of the aortic wall (given as a score as described in Subjects and methods)

Groups	WKY sham	WKY iso	WKY allo	WKY allo + ACEi	WKY allo + ET _A RB	WKY allo + comb	SHR sham	SHR iso	SHR allo	SHR allo + ACEi	SHR allo + ET _A RB	SHR allo + comb
Intima	0 \pm 0	0 \pm 0	2 \pm 0 ^a	1.2 \pm 0.45 ^{b,c}	1 \pm 0 ^b	0.6 \pm 0.55 ^b	0 \pm 0	0 \pm 0	2.4 \pm 0.55 ^a	2 \pm 0 ^a	1.6 \pm 0.55 ^b	1.4 \pm 0.55 ^b
Media	1.2 \pm 0.45	1 \pm 0	2.2 \pm 0.45 ^a	1.2 \pm 0.45 ^d	1 \pm 0 ^d	1 \pm 0 ^d	1.2 \pm 0.45	1 \pm 0	2.4 \pm 0.55 ^a	1.4 \pm 0.55 ^d	1 \pm 0 ^d	1 \pm 0 ^d
Adventitia	2 \pm 0	1.2 \pm 0.45 ^e	3 \pm 0 ^{e,f}	2 \pm 0 ^{f,d}	1.6 \pm 0.55 ^d	1.4 \pm 0.55 ^d	2 \pm 0	1 \pm 0 ^e	2.8 \pm 0.45 ^{e,f}	2 \pm 0 ^{f,d}	1.6 \pm 0.55 ^d	1.8 \pm 0.45 ^{d,g}

Sham, sham-operation; iso, isografting; allo, allotransplantation; ACEi, ACE inhibitor; ET_ARB, ET_A-receptor blocker; comb, combination therapy (ACEi + ET_ARB). ^a $P < 0.001$ vs sham and iso; ^b $P < 0.05$ vs sham, iso and allo; ^c $P < 0.05$ vs allo + comb; ^d $P < 0.05$ vs allo; ^e $P < 0.05$ vs sham; ^f $P < 0.05$ vs iso; ^g $P < 0.05$ vs iso.

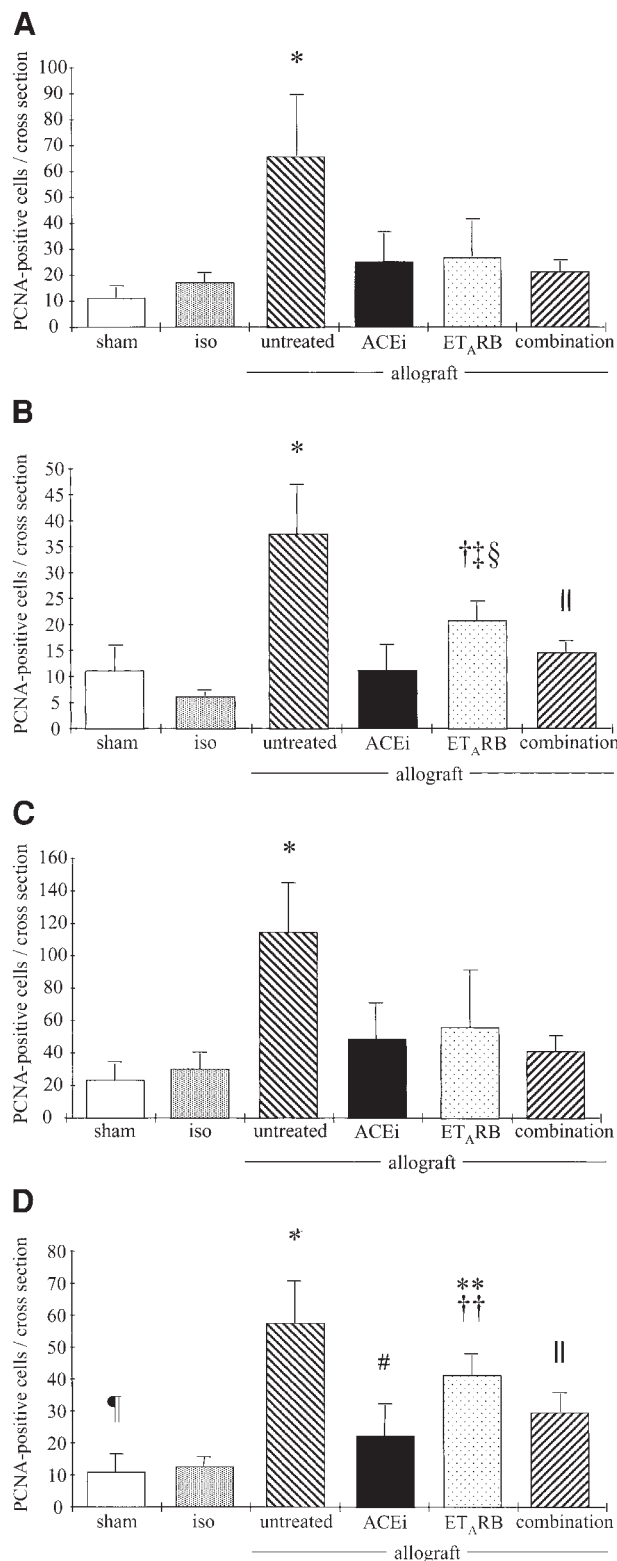


Fig. 4. PCNA-positive cells in the intima (A) and media (B) of sham-operated (sham), isografted (iso), and allografted (allo) WKY rats and SHR rats ((C) intima and (D) media), respectively. Allografted rats of both strains were treated with either trandolapril (ACEi) or LU 302146 (ET_ARB), or a combination of both. * $P < 0.001$ vs all groups; [†] $P < 0.001$ vs iso; [‡] $P < 0.05$ vs allo + ACEi; [§] $P < 0.01$ vs sham; ^{||} $P < 0.05$ vs iso; [¶] $P < 0.01$ vs allo + combination; [#] $P < 0.001$ vs allo + ET_ARB; ^{**} $P < 0.001$ vs iso and sham; ^{††} $P < 0.05$ vs allo.

are accepted standard models of chronic transplant vasculopathy [9]. Plissonnier *et al.* [10] reported that ACE inhibition is beneficial in these models. Therefore, we used the ACE inhibitor trandolapril as a positive treatment control. Interestingly, LU, in contrast to trandolapril, did not lower BP in the hypertensive model of WKY-to-SHR aorta transplantation, but it was nevertheless equally protective. On the other hand, both ACE inhibitor and ET_A-receptor blocker abrogated transplant vasculopathy in the normotensive model of SHR-to-WKY aorta transplantation despite no BP lowering.

The effect of LU must be interpreted to indicate that (i) ET plays a crucial role in progression of chronic transplant vasculopathy and (ii) that the effects of ETs are, at least partly, mediated via the ET_A receptor. The combination therapy of ACE inhibitor and ET_A-receptor blocker did not confer additive benefit on the parameters investigated. This finding is compatible with the idea that ACE inhibition and ET_A-receptor blockade share, at least in part, similar pathogenetic pathways. One possibility may be the reduction of ET-1 synthesis by ACE inhibition [11]. Another possibility may be that the undoubtedly present differences in the mechanisms of action of both drugs are equally effective in attenuating chronic transplant vasculopathy. Due to these differences one would have expected that combination therapy exerts an additive benefit. The lack of such an additive effect remains unclear, but it parallels the finding in the 'Fisher-to-Lewis' chronic allograft nephropathy model [5]. BP in SHRs was not lowered more by combination therapy than by ACE inhibition alone. This observation is of note, because some studies had reported that combination of angiotensin II receptor blockers with ET_A-receptor blockers had an additive hypotensive effect [12]. The lack of such an effect in our study may be due to the different animal models used, and the use of ACE inhibitors instead of angiotensin II receptor blockers.

Our experiment does not specifically exclude effects of ET_A-receptor blockade on immune recognition or effector steps in the allograft. There is a body of evidence, however, that ET-1 amplifies immune and non-immune effector mechanisms in chronic allograft rejection. The latter appears plausible in view of the known actions of ET-1 on vascular smooth muscle cells (VSMC). Transplant vasculopathy, the hallmark of chronic rejection, is characterized by endothelial cell damage and VSMC proliferation and migration. ET-1 is a potent mitogen for VSMC and mesangial cells [13], and the mitogenic effect of ET-1 is mediated via the ET_A-receptor [14].

Suppression of cell proliferation by ACE inhibition and ET_A-receptor blockade as a major beneficial mechanism is implicated by the results of our immunohistochemical studies. Treatment with either agent inhibited the increase in number of PCNA-positive cells in the media and intima of aorta allografts. Although PCNA is not an absolute specific marker of proliferation, this indicates attenuation of proliferative activity of aortic wall cells. As far

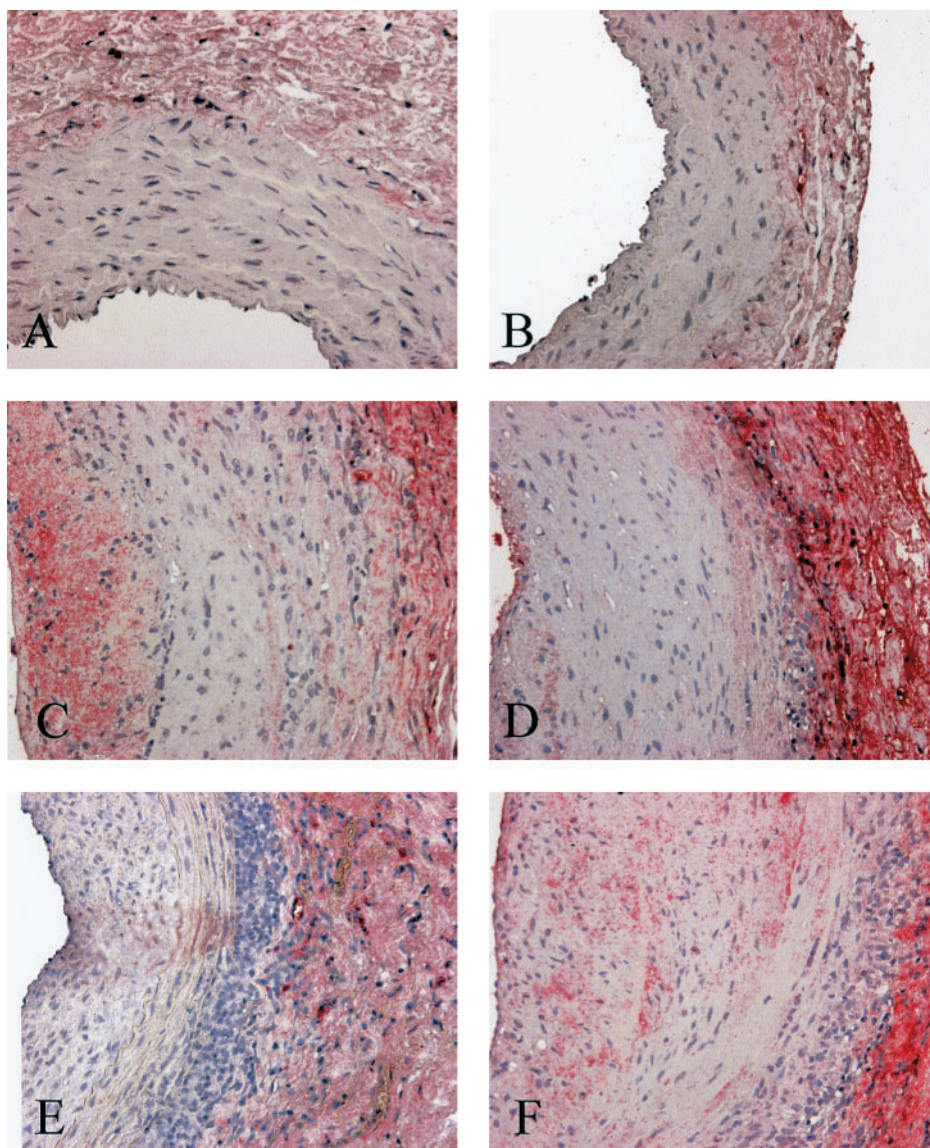


Fig. 5. TGF- β immunostaining in the different groups of SHR rats: sham-operated (A) and iso-transplanted (B) SHR animals as negative controls compared with untreated allotransplanted SHR (C), allotransplanted SHR treated with the ACE inhibitor trandolapril (D), the ET_A-receptor blocker LU 302146 (E), and a combination of ACE inhibitor and ET_A-receptor blocker (F), respectively. Magnification $\times 140$.

as the media is concerned, ACE inhibition appears to be more effective than ET_A-receptor blockade concerning this parameter. The reasons for this difference remain to be elucidated.

Interestingly, the expression of TGF- β was abrogated by ACE inhibition and combination therapy, but not by monotherapy with the ET_A-receptor blocker. This effect was particularly pronounced in the neointima. Overexpression of TGF- β is a known feature of chronic rejection in humans and animal models [11,15]. Abrogation of TGF- β immunostaining by ACE inhibition was also reported in the 'Fisher-to-Lewis' renal allograft model of chronic rejection [16]. TGF- β may be a player in chronic allograft vasculopathy because it is a strong growth factor promoting hypertrophy and extracellular matrix production of several cell types, e.g. VSMC [17]. Attenuation of TGF- β expression by

trandolapril is plausible due to activation of TGF- β by angiotensin II [18]. This indicates that the protective effect of trandolapril may be partially mediated by inhibition of TGF- β . On the other hand, the negative result of ET_A-receptor blockade on TGF- β immunostaining implies that the protective effect of LU is mediated via a different mechanism. TGF- β is also activated by ET-1, but the production of the latter is not affected by ET_A-receptor blockade [18], which may explain the negative result with LU. The lack of effect of LU on these parameters suggests other mechanisms of its beneficial effect on aortic graft thickening. One explanation may be that ET_A-receptor blockade suppresses the effect of other growth factors, such as platelet-derived growth factor [19] or epidermal growth factor [20]. Similarly to our results, Kelly *et al.* [18] reported that in the transgenic (mRen-2)27 rat, TGF- β

overexpression was not affected by the mixed ET_A/ET_B-receptor blocker bosentan, but by the angiotensin II receptor blocker valsartan.

We emphasize that we controlled several potential confounders. Specifically, sodium intake was similar in all groups and immunosuppressive treatment, i.e. cyclosporin A, was not used in the present study. This is of note, because cyclosporin A: (i) interacts with endothelial cell function; (ii) increases ET secretion from endothelial cells and VSMC; (iii) elevates ET plasma level; (iv) modulates ET-receptor expression; and (v) causes vasoconstriction.

Simonson *et al.* [4] reported increased immunoreactive ET-1 levels in the vasculature of chronic rejecting renal allografts in humans. This parallels earlier findings in coronary artery disease after heart transplantation: Ravalli *et al.* [3] documented increased ET-1 immunoreactivity in myointimal cells, macrophages and endothelial cells. Tanabe *et al.* [21] reported that endothelin-converting enzyme (ECE) is increased in arteries of human renal allografts with chronic transplant nephropathy, suggesting that ET-1 is generated from big ET-1 by ECE. Inhibition of ECE with phosphoramidon in a rat model of chronic cardiac allograft rejection attenuates transplant vasculopathy and rejection [22]. A preliminary report in an aorta allotransplantation model in the mouse documented abrogation of chronic transplant vasculopathy [23]. These findings together with the present study document that ET-1 plays a major role in the genesis of chronic rejection of different organs both in animals and humans. Thus, treatment of chronic rejection with specific ET_A-receptor blockers opens a new perspective.

In view of species differences of the vascular ET system, it is unknown whether the strikingly beneficial effects of selective ET_A-receptor blockade in the above aorta allotransplantation models can be extrapolated to humans. This question can only be addressed by prospective clinical trials.

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